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Review

Mitochondrial morphology in mitophagy and macroautophagy ☆

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ABSTRACT

Mitochondria are critical organelles in energy conversion, metabolism and amplification of signalling. They are however also major sources of reactive oxygen species and when dysfunctional they consume cytosolic ATP. Maintenance of a cohort of healthy mitochondria is therefore crucial for the overall cell fitness. Superfluous or damaged organelles are mainly degraded by mitophagy, a selective process of autophagy. In response to the triggers of mitophagy, mitochondria fragment: this morphological change accompanies the exposure of "eat-me" signals, resulting in the engulfment of the organelle by the autophagosomes. Conversely, during macroautophagy mitochondria fuse to be spared from degradation and to sustain ATP production in times of limited nutrient availability. Thus, mitochondrial shape defines different types of autophagy, highlighting the interplay between morphology of the organelle and complex cellular responses. This article is part of a Special Issue entitled: Mitochondrial dynamics and physiology.

1. Introduction

The word "autophagy", derived from the Greek and meaning "self-eating", was originally proposed by Christian de Duve more than 40 years ago to describe a catabolic process conserved from lower to higher eukaryotes [1]. Autophagy is essential for recycling energy sources when cells deal with challenging conditions, such as nutrient depletion or hypoxia, or during development [2]. Additionally, autophagy plays a key role in cellular quality control processes, being essential for the degradation of superfluous or damaged organelles and oxidized proteins [3].

Autophagy broadly refers to any process of degradation of cytosolic components by the lysosome, but it can be more precisely subdivided in 3 types identified based on the different cargo delivery to the lysosome — macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [4]. During macroautophagy, a "phagophore" expands into a double membrane organelle that engulfs cytosolic components (proteins, ribosomes and organelles), giving rise to the autophagosome. The external membrane of the autophagosome fuses with the lysosomal membrane, the inner vesicle and its cargo being therefore degraded. The ensuing nutrients are recycled back to the cytosol via membrane permeases [5]. Macroautophagy, the main focus of this review, will be hereafter referred as autophagy. Microautophagy differs from macroautophagy in that cytosolic components are directly sequestered by the lysosome through invaginations of the lysosomal membrane. In CMA, a form of autophagy described only in mammals, soluble proteins are delivered to the lysosome by crossing its membrane in a complex with chaperones.

Initially, autophagy was believed to be a non-selective process, meaning that cytosolic components would be randomly surrounded by the autophagosome. Albeit de Duve in 1966 suggested that autophagy could be selective, data supporting this hypothesis were lacking at the time [6]. Later on, under specific conditions, certain macromolecular components were found to be preferentially delivered to the lysosome [7–9]. Several examples of selective degradation have been then revealed, including the specific break down of aggregated proteins [10], the selective removal of superfluous or damaged organelles — like mitochondria (mitophagy) [11], peroxisomes (pexophagy) [12] and endoplasmatic reticulum (ER-phagy) [13] — and the specific degradation of invading bacteria (xenophagy) [14]. Selectivity in cargo targeting to the autophagosome is mediated by autophagy receptors, proteins that simultaneously interact with specific cargoes and with autophagy modifiers conjugated to the autophagosomal membrane, like yeast Atg8 and the mammalian homologues LC3/GABARAP proteins [15,16].

Abbreviations: ATG, autophagy-related genes; CCCP, cyanide m-chlorophenylhydrazone; CMA, chaperone-mediated autophagy; CsA, cyclosporine A; $\Delta\psi_m$, mitochondrial membrane potential; IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; PAS, phagophore assembly site; PTP, permeability transition pore; ROS, reactive oxygen species; RTG, retrograde signalling pathway

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The term "mitophagy" was introduced by Lemasters in 2005 [17], even if the first descriptions of mitochondria inside lysosomes date from circa 40 years before. Engulfment of mitochondria together with other organelles by lysosomes in rat hepatocytes exposed to glucagon was described in 1962 [18]. Moreover, in 1977, Beaulaton and Lockshin described that during metamorphosis of silkmoth muscles, autophagy targeted almost exclusively mitochondria, the first example of selective mitochondrial autophagy [8]. In the last few years, mitophagy has been intensively studied. Accumulating evidence indicates that mitochondria can be selectively removed by autophagy and the signals that specifically target mitochondria to autophagy have started to be unravelled.

Mitochondria are dynamic organelles that continuously fuse and fragment during cell life, appearing in situ as short round-shaped or elongated organelles, with a major axis that can reach 5 μm [19]. On the other hand, autophagosomes are globular organelles with a diameter of approximately 1 μm [3], posing a sterical problem to mitochondrial engulfment by autophagosomes. Indeed, it has been suggested that mitochondrial fragmentation precedes mitophagy [20–23]. Conversely, when massive autophagy is induced in the cells by nutrient depletion, for instance, mitochondria elongate [24,25]. Elongated mitochondria are spared from autophagy and optimize ATP production in times of starvation [24].

In this review, we provide an overview of the molecular mechanisms of mitophagy, in yeast and mammals, focusing on the relationship between autophagy and mitochondrial dynamics and on the different features of mitochondrial shape during mitophagy and macroautophagy.

2. Mitophagy

Mitochondria are crucial organelles for energy production, regulation of cell signalling and amplification of apoptosis [26–28]. At the same time however, they are the major source of reactive oxygen species (ROS) that may oxidize mitochondrial own lipids, proteins and DNA [29]. Therefore, mechanisms of mitochondrial quality control have evolved to avoid cell damage and to maintain the overall fitness of the cell. Mitophagy has emerged as a key mechanism in this quality control, responsible of the elimination of superfluous or damaged mitochondria [30]. The critical role of autophagy in the maintenance of a "healthy" cohort of mitochondria was shown both in yeast [31] and mammals [23]. Yeast strains carrying deletions in autophagy-related genes (*ATG*) are unable to degrade mitochondria during the stationary phase, display growth defects in non-fermentable carbon sources and accumulate dysfunctional mitochondria. Accordingly, *ATG* mutants show lower oxygen consumption rates, decreased mitochondrial membrane potential and higher ROS levels [31]. Similarly, maximal respiration is reduced in mammalian cells deficient for ATG5 or treated with a pharmacological inhibitor of autophagy [23]. These genetic evidences support an essential role for mitophagy in the maintenance of mitochondrial and therefore cellular health. We will now overview our current knowledge of how mitophagy is triggered in yeast and mammalian cells, highlighting the relationship between this degradation process and the shape of the organelle.

2.1. Mitophagy in yeast

2.1.1. When?

The first studies in *Saccharomyces cerevisiae* provided evidence that in order to be targeted to autophagy, mitochondria need to be dysfunctional [32,33]. Priault and colleagues found increased mitophagy under anaerobic conditions in a *FMC1* null mutant, where the mitochondrial ATPase is dysfunctional. During anaerobiosis, the ATPase by operating in reversal (i.e., by hydrolyzing ATP) maintains mitochondrial membrane potential ($\Delta\psi_m$), and, consequently, mitochondrial ion homeostasis and biogenesis. In *FMC1* null strain during anaerobiosis, $\Delta\psi_m$ collapses since mitochondria cannot use glycolytic ATP to maintain $\Delta\psi_m$. The authors proposed that this defect targets mitochondria to autophagy [33]. The idea that mitochondrial dysfunction leads to the removal of the organelle was further supported by Nowikovsky and colleagues [34]. Shutting-off the expression of *MDM38* leads to loss of mitochondrial K^+/H^+ exchange, osmotic swelling, reduction of $\Delta\psi_m$, mitochondrial fragmentation, and, mitophagy. Even though mitochondrial dysfunction targets the organelle to autophagy, treatment of yeast with the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), that dissipates $\Delta\psi_m$ is not sufficient to induce mitophagy [35,36], suggesting that an additional yet unidentified factor is required [37].

Induction of non-selective macroautophagy also leads to mitophagy in yeast [36,38,39]. Indeed, mitophagy can be induced by nitrogen starvation or by the Tor kinase inhibitor rapamycin in yeast previously grown in a non-fermentable carbon source that induces mitochondrial proliferation. Nevertheless, macroautophagy and mitophagy appear to be differentially regulated: nitrogen-starvation in the presence of a non-fermentable carbon source induces macroautophagy, but not mitophagy. It should be stressed that under these metabolic conditions mitochondria are essential for energy production [39], highlighting the existence of signalling cascades that can spare mitochondria from autophagic degradation. One interesting possibility, as we will discuss later, is that sterical hindrance of elongated organelle from engulfment by the autophagosomes prevents mitophagy when mitochondria are required for energy production. Along this line, superfluous mitochondria in yeast are also removed by mitophagy: mitochondrial removal is induced during stationary phase in a non-fermentable carbon source [38–40], when energy requirements are reduced.

2.1.2. How?

The search of a specific signal targeting mitochondria to autophagosomes has been intensive and led to the description of the first mitochondrial "eat me" signal in yeast in 2004. Uth1, an outer mitochondrial membrane protein, has been found to be essential for mitophagy induced by rapamycin or nitrogen starvation, without affecting per se the autophagic machinery [35]. Few years later, in a screen for functional interactors of Atg1, the mitochondrial protein Aup1 was identified to be essential for efficient mitophagy during stationary phase. Under these conditions, and in disagreement with the study of Kissova and co-workers, mitophagy played a pro-survival role, since deletion of Aup1 was lethal [38]. It has been recently suggested that Aup1 regulates mitophagy by controlling the retrograde signalling pathway (RTG) [41]. Nevertheless, the function of both Uth1 and Aup1 in mitophagy has been challenged by Kanki and colleagues [42]. In their hands, lack of these proteins did not block mitophagy, possibly as a consequence of the differences in the background of the yeast strains used or in the detection methods.

Recently, in a genomic screen for yeast mutants defective in mitophagy, two other mitochondrial proteins have been identified, named Atg32 and Atg33 [36,40,42]. Atg32 is an outer membrane protein, essential for mitophagy, but not for macroautophagy or other types of selective autophagy. Selective autophagy in yeast requires a receptor and an adaptor protein: Atg32 acts as a receptor protein that interacts with the adaptor protein Atg11, most likely to sequester mitochondria to the phagophore assembly site (PAS) [40,42]. In addition, Atg32 possesses an evolutionary conserved motif (WXXI/L) critical for the interaction with Atg8, an ubiquitin-like protein essential for autophagosomal membranes growth. The interaction between Atg32 and Atg8 is required for mitochondrial recruitment by the phagophore [40]. Atg32has been the first protein described to be required to mitophagy and to interact with the autophagic machinery. Nonetheless, important questions remain open: what is the physiological significance of Atg32-induced mitophagy? In other words, what happens in the absence of Atg32? Surprisingly enough, no differences

were observed between wt and the strain lacking Atg32 when cell growth was analysed for 3 days in a non-fermentable carbon source, or when cell viability and ROS production under starvation conditions were examined, a striking difference with the mitochondrial defects detected in *ATG* deleted strains [31]. In addition, mitochondrial mass and DNA levels were not affected by ablation of Atg32, ruling out a critical role for this protein in the removal of superfluous mitochondria. Conversely, Atg32 seems to be critical for mitophagy induced by the ablation of *MDM38*, when mitophagy has been proposed to take place in response to mitochondrial damage [42]. Atg32 levels increase in mid-log phase under respiratory conditions, decreasing through late to post-log phase, suggesting that Atg32 is upregulated before the onset of mitophagy. Yet, Atg32 is expressed also in conditions that do not lead to mitophagy, indicating that other factors must be involved in promoting it [40]. Indeed, upon induction of mitophagy Atg32 is phosphorylated at serine 114 to allow the Atg11–Atg32 interaction and therefore the targeting of mitochondria to the autophagosomes. Atg32 phosphorylation seems to be carried on by MAPKs described to be involved in mitophagy, Slt2 and Hog1 [43], shedding light on the signalling cascade that might be operative to trigger mitophagy [44].

Another protein retrieved in the screening of yeast mutants deficient in mitophagy is Atg33 [36]. Lack of Atg33, an outer mitochondrial membrane protein, blocks mitophagy during nitrogen starvation and abolishes it almost completely during the stationary phase. The preponderant role of Atg33 during stationary phase suggests that Atg33 is probably required for the recruitment of aged mitochondria by the PAS. Again, however, functional studies to prove this hypothesis are lacking.

2.2. Mitophagy in mammals

2.2.1. When?

At a major difference from yeast, where the role of mitochondrial dysfunction and depolarization in triggering mitophagy is still a matter of debate, in mammals it is well documented that the loss of $\Delta\psi_m$ causes mitophagy. For instances, mitochondria are selectively removed upon treatment with the uncoupler CCCP [45,46]. Lemasters and colleagues have supported a model in which opening of the mitochondrial permeability transition pore (PTP), an inner mitochondrial membrane unselective channel [47] triggers mitophagy [48]. Starvation and treatment of hepatocytes with glucagon lead to PTP opening, mitochondrial depolarization and removal. Although under these conditions few mitochondria seemed to be removed by autophagy, the ones where PTP opening occurred were selectively eliminated. Inhibition of the PTP by the immunosuppressant cyclosporine A (CsA) reduced also the proliferation of autophagosomes. However, no direct measurements of mitophagy were performed in hepatocytes where the PTP was inhibited [11]. Additionally, CsA is a powerful inhibitor of mitochondrial fragmentation caused by depolarization of the organelle, acting at the level of calcineurin dependent mitochondrial translocation of the mitochondrial fission protein DRP1 [49]. While Lemasters and colleagues ruled out a role for calcineurin in autophagosome proliferation, they did not measure whether CsA or calcineurin were directly involved in mitophagy [11]. An intriguing alternative hypothesis could be that CsA could interfere with mitophagy not at the level of PTP, but by altering mitochondrial shape, as we will see below. In accordance with the idea that mitochondrial dysfunction triggers mitophagy, neurons deprived of nerve growth factor in the presence of caspase inhibitors, selectively lose their mitochondria in a process that involves autophagy and that is blocked by the anti-apoptotic molecule BCL-2 [50].

Mitochondria are also removed by autophagy during differentiation of specific cells, like reticulocytes that differentiate into red blood cells that completely lack mitochondria (and other organelles). Genetic evidence support a crucial role for NIX, a pro-apoptotic BH3-only member of the BCL-2 family, in the selective targeting of mitochondria to the autophagosome (see below for details) [46,51]. Additionally, mitophagy has been reported to play a crucial role during T-lymphocytes development [52]. Thus, mitophagy is specifically triggered during differentiation of specific tissues, when it is needed for their maturation. We can foresee that the analysis of these specific mechanisms of mitophagy may also contribute to elucidate the molecular mechanisms of mitophagy of dysfunctional mitochondria.

2.2.2. How?

Insights into the mechanism triggering mitophagy came from the analysis of developmental mitophagy: as we said before, mitochondrial clearance is a key step during reticulocyte maturation. This process likely occurs through autophagy since ablation of autophagy specific genes (ULK1 and ATG7) impairs mitochondrial removal [53,54]. NIX, also known as BNIP3-like protein (BNIP3L), is crucial for the complete removal of mitochondria during reticulocyte development [46,51]. Expression of NIX increases during the terminal stages of erythroid differentiation [55] and $Nix^{-/-}$ mice are anaemic with a compensatory expansion of erythroid precursors. Strikingly, $Nix^{-/-}$ red blood cells retain mitochondria but not other organelles [46,51]. The mechanism by which NIX participates in mitochondrial clearance is still a matter of debate. NIX has been proposed to be a selective autophagy receptor for mitophagy [56], like Atg32 in yeast. Accordingly, the conserved LC3-interating region (LIR) of NIX interacts with the Atg8 homologues LC3/GABARAP [56,57], ubiquitin-like proteins essential for autophagosomal membranes growth. If the interaction between NIX and LC3/GABARAP is impaired, mitochondrial removal in mouse reticulocytes is delayed but not blocked, suggesting that other features of NIX from the classical core autophagic machinery are important [53,54,58]. Additionally, complete recruitment of GABARAP-L1 to damaged mitochondria requires the integrity of the LIR domain of NIX [56]. On the other hand, induction of depolarization can restore mitochondrial clearance in NIX deficient erythrocytes, suggesting that mitochondrial depolarization targets mitochondria to autophagy in a NIX-independent manner [46].

NIX was originally cloned based on its homology to BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) [59], another mitochondrial outer membrane protein with a LIR region homologous to the NIX one [56]. BNIP3 also interacts with LC3 [60] and is required for hypoxia-induced mitophagy [61]. The possibility that BNIP3 acts as an autophagy receptor for mitochondria remains however to be fully explored. Recently, FUNDC1, an OMM protein, was found to mediate hypoxia-induced mitophagy, through interaction of its LIR domain with LC3. Under hypoxic conditions, FUNDC1 gets dephosphorylated at Tyr 18, increasing its binding to LC3-II [62].

Mitophagy in $Nix^{-/-}$ reticulocytes can be restored by uncoupling of mitochondria with CCCP, suggesting that other pathways exist for the removal of damaged mitochondria. Pioneering work from Youle and colleagues provided evidence that PARKIN, an E3 ubiquitin ligase mutated in autosomal recessive forms of Parkinson's disease, translocates from the cytosol to mitochondria in cells treated with CCCP or with the herbicide paraquat that increases ROS formation. After recruitment, PARKIN mediates selective engulfment of depolarized mitochondria by autophagosomes [45]. PINK1, a mitochondrial kinase, that is also mutated in autosomal recessive forms of Parkinson's disease, seems to be required for PARKIN recruitment to impaired mitochondria both in mammals [63–66] and in *Drosophila melanogaster* [67]. In a widely accepted model, endogenous PINK1 is rapidly degraded in healthy mitochondria, whereas it accumulates on the surface of damaged, depolarized organelles as a consequence of an impairment of protein import, driving phosphorylation of PARKIN and therefore its translocation to mitochondria [65]. Ubiquitin has been proposed to act as a signal for selective autophagy in mammalian cells. Different cargos (protein aggregates, ribosomes, peroxisomes and pathogens) are ubiquitinated before being removed by

autophagy [15]. After translocation to the depolarized mitochondria, PARKIN catalyzes poly-ubiquitination of several substrates, calling into play the autophagy receptor p62/SQSTM1 that simultaneously binds ubiquitin and autophagy-specific ubiquitin-like modifiers (LC3/GABARAP proteins). However, the role p62 in PARKIN-induced mitochondrial clearance remains controversial: contrasting reports deem it indispensable [63,68] or not [69,70] for mitophagy. The observed differences may be explained by functional redundancy, since lack of p62 could be compensated by a different autophagy receptor that binds both ubiquitin and LC3/GABARAP proteins, like NBR1 [71]. After ubiquitination and recruitment of p62, depolarized mitochondria are transported along microtubules to the perinuclear region, where they form "mito-aggresome" structures [66,68,70]. Ubiquitinated mitochondria recruit not only p62 but also HDAC6, a ubiquitin-binding protein deacetylase that mediates transport of damaged mitochondria, facilitating their clustering at the perinuclear region for subsequent clearance [68]. PARKIN participates in ubiquitination of the mitochondrial fusion proteins MFN1 and MFN2 [72,73], and of MARF, the *Drosophila* orthologue [67]. MFNs, however, are not essential for PARKIN-dependent mitophagy, since this process was observed in *Mfn1*$^{-/-}$*Mfn2*$^{-/-}$ MEFs [45]. In addition, following recruitment of PARKIN to damaged mitochondria, Mfns are lost prior to mitophagy [67,72,73], being degraded in a proteasome and p97-dependent manner [73]. Hence, ubiquitination of mitofusins does not probably constitute a signal for mitophagy, but their removal by the proteasome can promote mitochondrial fragmentation, important for subsequent engulfment by the autophagosomes, as we will discuss later. In addition to these mitochondria-shaping and adaptor proteins, following mitochondrial depolarization AMBRA1, a component of the core autophagic machinery [74], interacts with PARKIN to mediate mitochondrial clearance [75], suggesting that PARKIN can directly cross-talk to the autophagic machinery. PARKIN is not the only E3 ubiquitin ligase found on mitochondria: MARCH 5/MITOL or MULAN belong to the same family and are associated with the mitochondrial outer membrane, opening the interesting possibility that they might be involved in mitophagy [76].

PARKIN-induced autophagy has been studied in the context of mitochondrial damage. On the other hand, the role of NIX in mitophagy has been studied mainly during reticulocyte maturation. Interestingly, the interaction between NIX and GABARAP-L1 seems to play an important role also in cell types other than reticulocytes [56]. For example, NIX can promote PARKIN translocation to depolarized mitochondria in MEF cells [77]. Do NIX and PARKIN participate in the same pathway to induce mitophagy? Or do different proteins play a role in mitophagy depending on the inducing conditions or the cell type? These and other open questions await experimental answers. A cartoon summarizing our current knowledge of molecules involved in mitophagy in yeast and mammalian cells is shown in Fig. 1.

3. Mitochondrial morphology in mitophagy and autophagy

3.1. Mitochondrial fusion and fission

Mitochondria are highly dynamic organelles and their morphology, size and distribution are precisely controlled, in order to meet cellular requirements. Mitochondrial dynamics is regulated by mitochondrial-shaping proteins. As one could expect, considering the crucial role of mitochondria, this family of proteins has been implicated in diverse cellular functions, such as calcium signalling [78], formation of dendritic spines [79], migration of lymphocytes [80], cell cycle [81], apoptosis [82,83] and even lifespan in lower eukaryotes [84]. Mitochondrial shape depends on the balance between two processes that occur continuously during cell life — fission and fusion. When either of them is blocked, mitochondrial morphology is the outcome of the unopposed progression of the other one [85].

The core mitochondrial fission machinery in mammals is constituted by the cytoplasmic dynamin-related protein 1, DRP1 [86] and the outer mitochondrial membrane (OMM) proteins, FIS1 [87] and MFF [88]. DRP1 is a large GTPase with structural homology to dynamin that participates in fission of both mitochondria and peroxisomes [89]. DRP1 requires to be translocated to the mitochondria to drive mitochondrial fission, being its localization controlled by post-translational events. Rise of cytosolic Ca^{2+}, associated with mitochondrial depolarization, leads to DRP-1 dephosphorylation by calcineurin at serine 637 and concomitant translocation of DRP1 to mitochondria [49], where it is stabilized by sumoylation [90] and participates in fission. Conversely, protein kinase A (PKA) phosphorylates DRP1 at serine 637, restraining fission [91,92]. Alternatively, DRP1 can be phosphorylated also at serine 637 by calcium/calmodulin-dependent protein kinase Ialpha (CAMKIα) [93] or at serine 616 by cyclin-dependent kinase 1 (CDK1) [94]. However, when phosphorylated by these two kinases, DRP1 drives mitochondrial fission. FIS1 is a transmembrane protein with a small region facing the intermembrane space (IMS) and two tetratricopeptide-repeat (TPR)-like domains in the cytosol. There is evidence that FIS1 acts as an adaptor for DRP-1 in OMM [95]; however, whether FIS1 is absolutely needed for DRP-1-dependent fission is not clear. MFF is an integral protein of the OMM that has been recently reported to participate in mitochondrial fission, by recruiting DRP-1 to mitochondria in a FIS1-independent fashion [88]. Accordingly, MFF and FIS1 are retrieved in two separate high molecular weight complexes [96]. Orthologues of DRP1 and FIS1 in yeast (Dnm1 and Fis1) are structurally similar; however, an adaptor protein, Mdv1, is required for efficient fission of yeast mitochondria [97].

The main regulators of mitochondrial fusion are the dynamin-like GTPases MFN1 and MFN2 in the OMM [98] and OPA1 in the inner mitochondrial membrane (IMM) [99]. Although MFN1 and MFN2 display a high degree of homology, the GTPase activity of MFN1 is

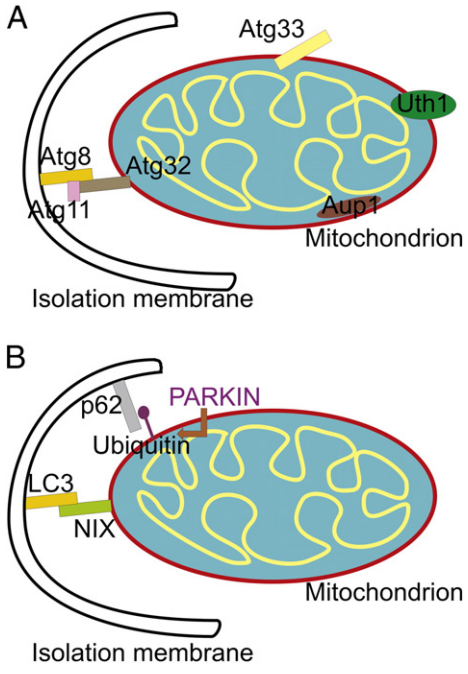

Fig. 1. Signals that target mitochondria to autophagy. (A) In yeast, four mitochondrial proteins have been reported to be important for targeting mitochondria to autophagy. Atg32 directs mitochondria to the autophagosome through its interaction with both Atg11 and Atg8. The mechanism by which Uth1, Aup1 and Atg33 target mitochondria to the autophagosome is not clear. (B) In mammals, during erythrocyte differentiation, NIX targets mitochondria to the autophagosome through its interaction with LC3. Upon mitochondrial depolarization, PARKIN ubiquitinates proteins in the OMM. Whether ubiquitinated proteins target the mitochondria to the autophagosome by their interaction with p62 is still unclear.

much higher, making these proteins functionally different. Accordingly, MFN1, but not MFN2, is required by OPA1 to promote mitochondrial fusion [99]. MFN2, on the other hand, has been implicated in other cellular functions such as cell metabolism [100], cell proliferation [101] and ER-mitochondria tethering [102]. In addition, *Mfn2* mutations are associated with the peripheral neuropathy Charcot–Marie–Tooth disease type 2A [103]. The activity of the IMM profusion protein, OPA1, is regulated by proteolytic cleavage [104] and both long and short forms of OPA1 are needed for fusion [105]. Besides its role in mitochondrial fusion, OPA1 is also essential for IMM structure [106,107] and for apoptosis, by keeping in check the size of the cristae junctions [107,108] that enlarges to allow cytochrome *c* release during cell death [83]. Interestingly, the functions of OPA1 are impaired by mutations of the protein associated with autosomal dominant optic atrophy [109,110].

Besides the canonical mitochondrial fission and fusion proteins, other molecules have been proposed to regulate mitochondrial shape. Endophilin B1 [111], mitochondrial protein 18 kDa (MTP18) [112], mitofusin-binding protein (MIB) [113] and ganglioside-induced differentiation-associated protein 1 (GDAP1) [114] seem to be involved in mitochondrial fission. Conversely, leucine zipper-EF-hand containing transmembrane protein1 (LETM1) [115], phospholipase D (PLD) [116] and prohibitins [117] are required for mitochondrial fusion.

3.2. Mitochondrial fission is a pre-requisite for mitophagy

Accumulating evidence emphasizes the requirement of mitochondrial fragmentation prior to mitophagy [20–23,34,36]. Conceptually, this is not surprising given that an organelle that is going to be engulfed and degraded by the autophagosome needs to fit into this forming structure. Considering that individual mitochondrial length averages 5 μm, and that autophagosomes display a diameter of around 1 μm, a role for mitochondria-shaping proteins in solving this sterical hindrance could have been anticipated.

Mitochondrial fission and mitophagy often co-exist: for example, in response to nitric oxide (NO), in primary cortical neurons mitochondria undergo fission and are retrieved inside autophagosomes [118]. A similar connection was found in models of Alzheimer's disease, where mitochondrial fragmentation [119] is accompanied by an increase in the number of mitochondria found in autophagosomes [120]. However these correlative studies do not answer to the question of whether mitochondrial fission is required for mitophagy. Arnoult and colleagues showed that induction of apoptosis triggers mitophagy, that is preceded by mitochondrial fragmentation. Inhibition of mitochondrial fission also impairs mitophagy, suggesting a role for the shape of the organelle in the process [20]. Accordingly, when mitochondrial fission is chronically blocked, mitochondria become dysfunctional and levels of autophagy increase. Nonetheless, mitophagy is not observed, suggesting that even if mitochondria were damaged, they were probably too long to be engulfed by the autophagosome [22]. In yeast, mitophagy in the *MDM38* δ strain is preceded by mitochondrial fragmentation [34] and *DNM1* was found in a genetic screen for yeast mutants defective in mitophagy. Deletion of *DNM1* did not completely block mitophagy, although it inhibited it partially [36]. Recently, however, it was reported that rapamycin-induced mitophagy in yeast is independent of mitochondrial fission, questioning the requirement for mitochondrial fission machinery during mitophagy in yeast [121]. On the other hand, in mammals mitophagy was consistently shown to be accompanied by fragmentation of the organelle. Do these circumstantial evidences mean that mitochondrial fission is per se sufficient to induce mitophagy? We reported that high levels of the mitochondrial fission protein FIS1 induce autophagy. However, in this model mitochondrial dysfunction, a well characterized consequence of FIS1 overexpression [122], rather than fission triggered autophagy. Indeed, over-expression of a mutant of FIS1 that induces mitochondrial fragmentation, but not dysfunction, was not associated with increased autophagy and mitophagy [21]. Autophagy induced by FIS1 overexpression appeared not to be selective for mitochondria, as it resulted from the activation of AMPK both *in vitro* and *in vivo* [123]. However, upon enforced FIS1 expression, some of the fragmented and dysfunctional mitochondria were retrieved into autophagosomes, further suggesting that when fragmented, dysfunctional mitochondria can be easily targeted by autophagy [21].

The data discussed above show that while mitophagy is invariantly preceded by mitochondrial fission, fragmentation per se does not seem to represent a signal to target mitochondria to the autophagosome. Thus, the question of how dysfunctional mitochondria are segregated from the network in order to be degraded remained open until an elegant study by Shirihai and colleagues provided evidence for a selection process that filters "good" from "bad" mitochondria targeted to autophagy. In pancreatic β-cells, mitochondria undergo frequent cycles of fusion followed by fission. Often a fission event gives rise to uneven daughter mitochondria in respect to their membrane potential: one displays high $\Delta\psi_m$, the other low $\Delta\psi_m$ and has a reduced probability to fuse. This population of fragmented mitochondria with decreased $\Delta\psi_m$ and lower levels of OPA1 (that is degraded in depolarized mitochondria) is removed by autophagy. Blocking fission, however, impaired mitophagy, resulting in the accumulation of dysfunctional mitochondria [23]. Mitochondrial components are believed to freely mix through fusion of the mitochondrial network. Indeed, mitochondrial mutations have been shown to spread across the network and exchange of gene products allows complementation of function [124]. The work by Twig and colleagues, however, shows that mitochondrial fusion is not an unselective process — the probability of mitochondria with lower $\Delta\psi_m$ to fuse is reduced [23]. mtDNA mutations do not inevitably lead to a decrease in $\Delta\psi_m$ and the half-time of respiratory complexes is longer than the latency between fusion events. The combination of these factors could explain the long latency needed to acquire a bioenergetic phenotype [125] and how fusion can work in complementing mtDNA mutations. If this was the case, one could predict that inhibition of fusion can improve mitochondrial quality: supporting this possibility, long-term over-expression of PARKIN that prevents re-fusion of damaged mitochondria promotes elimination of mitochondria with COXI mutations in heteroplasmic cybrids [126].

In conclusion, mitophagy requires efficient fission that helps segregating the bad organelles and prepares them to fit into the autophagosomes. However, fission per se is not the trigger of mitophagy, for which a concomitant dysfunction of the organelle, or other yet unclear signals, are required.

3.3. Mitochondria elongation during macroautophagy: avoiding mitophagy to sustain ATP production

Mitochondria are not just innocent bystanders during macroautophagy: they might play a role in autophagosomal biogenesis, by providing membranes for the formation of the PAS [127], in a process that depends on the tethering to the endoplasmic reticulum [102]. Moreover, ROS, mainly produced by mitochondria during starvation, have been reported to play an essential role as regulators of autophagy [128].

A conundrum is whether also mitochondrial shape varies when autophagy is induced, similarly to what occurs during apoptosis [82,83]. A simple guess would be that mitochondria fragment in order to be degraded. We set out to address this question and we surprisingly found that after induction of macroautophagy by starvation or mTOR silencing, mitochondria elongate both *in vitro* and *in vivo* [24]. Starvation leads to an increase in cyclic AMP (cAMP) levels and therefore to PKA activation. PKA phosphorylates serine 637 of DRP1, thereby preventing its mitochondrial relocalization and inhibiting fission. Mitochondrial elongation during induction of

macroautophagy results from unbalanced fusion, as substantiated by a genetic screening that identified the core fusion machinery as a requisite for mitochondrial elongation during macroautophagy. A simple prediction based on the sterical requirements described above for mitophagy, the elongated mitochondria produced in response to macroautophagy are spared from degradation. In addition, elongated mitochondria possess more cristae where activity of the ATP synthase is increased, optimizing ATP production. When elongation is blocked genetically or pharmacologically, mitochondria conversely consume ATP, precipitating starvation-induced death. Interestingly, fragmentation also leads to mitophagy during starvation, raising the question of whether mitochondria expose an "eat me" signal as a mere consequence of starvation, or whether fragmentation is per se sufficient to drive mitophagy under conditions of limited nutrient availability. Nevertheless, our results indicate that mitochondrial shape determines the cellular fate during macroautophagy, extending the role of mitochondria as key signalling organelles also to this paradigm of reversible cellular response [24]. Our results have been confirmed by an independent study where elongation was also found to be dependent on the nutrient being depleted [25]. Accordingly, essential amino acids can prevent starvation induced elongation, but not revert it, suggesting that intricate networks exist to control the response of the cell to the type of nutrient depleted [129].

In conclusion, mitophagy and macroautophagy can be separated not only based on their different triggers, molecular mechanisms, consequences, but also based on the mitochondrial morphologies that characterize them: mitophagy is preceded by organelle fragmentation, while autophagy is accompanied conversely by their elongation (Fig. 2). These two shapes are not random, as they ensue from specific molecular mechanism impinging on different components of the mitochondria shaping machinery. Future research will elucidate the fine mechanisms of cross-talk between autophagy and mitochondrial shape to define both cellular and mitochondrial quality control programs.

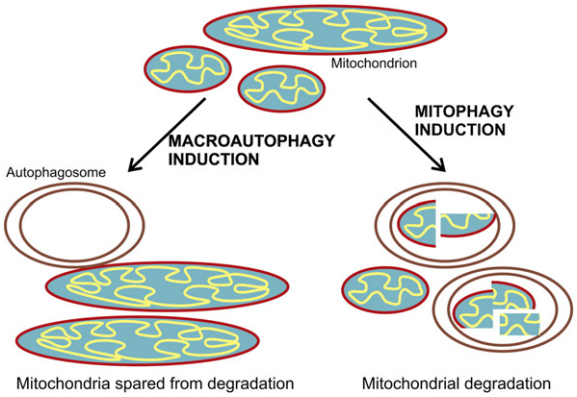

Fig. 2. Mitochondrial shape is different in mitophagy and macroautophagy. During macroautophagy, mitochondria elongate and are spared from degradation. Conversely, selective degradation of mitochondria by mitophagy is preceded by mitochondrial fragmentation.

Authors' contributions

LCG and LS wrote the manuscript.

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